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Apoptosis in Pulmonary Fibrosis: Too Much or Not Enough?

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ABSTRACT

Apoptosis plays an important role in both normal lung homeostasis and lung remodeling associated with fibrotic lung disease. Whether apoptosis promotes or inhibits the pathogenesis of pulmonary fibrosis depends upon the cell type involved and the microenvironment of the affected lung. Undue cell loss in the alveolar epithelium may be important early in idiopathic pulmonary fibrosis (IPF) progression, while reduced fibroblast and myofibroblast apoptosis has been associated with the formation of fibrotic lesions. As such, novel therapies based on the stimulation or inhibition of apoptosis may prove beneficial to the treatment of patients with IPF. *Antioxid. Redox Signal*. 10, 379–385.

INTRODUCTION

An increasing body of evidence outlines an important role for apoptosis in both normal lung homeostasis and the pathogenesis of a variety of lung diseases. A number of studies now support a role for apoptosis in lung remodeling associated with fibrotic lung diseases. In fact, this process appears to be an essential feature of idiopathic pulmonary fibrosis (IPF). Whether apoptosis promotes or inhibits the pathogenesis of IPF may depend upon the cell type involved and the microenvironment of the affected lung. Undue cell loss in the alveolar epithelium is thought to be an important early event in IPF progression, while reduced levels of apoptosis of fibroblasts and myofibroblasts have been associated with the formation of fibrotic lesions. As such, novel therapies based on the stimulation or inhibition of apoptosis may prove beneficial to the treatment of patients with IPF.

OXIDATIVE STRESS AS MEDIATOR OF PULMONARY FIBROSIS

A current theory regarding the pathogenesis of IPF is that in the alveolar regions of affected lungs there exists an imbalance between oxidant production and the level of antioxidant protection (22). It is well documented that a wide variety of oxidants are produced under pathological conditions that lead to pulmonary fibrosis (reviewed in Ref. 13). Both exogenous and endogenous sources of reactive oxygen species (ROS) exist in the diseased lung, including key enzymatic mediators such as myeloperoxidase, and cellular mediators such as alveolar macrophages, neutrophils, and fibroblasts (2). To combat these destructive oxidants, the lung contains highly specialized and compartmentalized antioxidant defenses such as glutathione and other small molecular weight antioxidants, as well as antioxidant enzymes such as catalase and the superoxide dismutases (9, 12). Induction of these antioxidant proteins after pulmonary insults may protect the lung and promote repair. Conversely, impaired induction or inactivation/clearance of antioxidant enzymes may result in a sustained redox imbalance that contributes to the progression of pulmonary fibrosis.

The consequence of a sustained redox imbalance is ultimately the disruption of normal lung tissue homeostasis. Increased levels of oxidants result in abnormally high levels of alveolar epithelial cell (AEC) death, often by apoptosis, leading to the denuding/destruction of the alveolar basement membrane. In an effort to repair this damage, fibroblasts are recruited to the site of damage and proceed to secrete extracellular matrix (ECM) components to provide temporary scaffolding upon which the alveolar epithelium can be reassembled. In instances of normal repair, once the epithelium is restored, the ECM is reabsorbed

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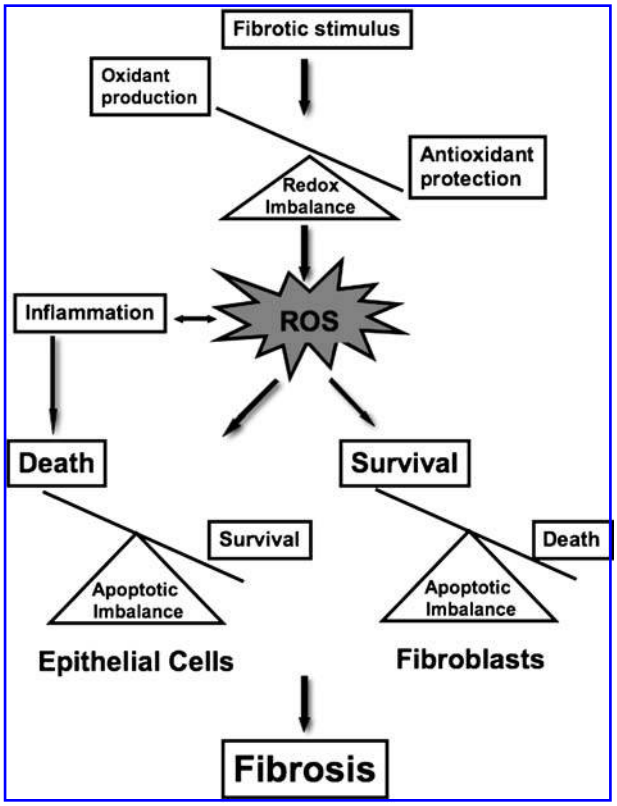

FIG. 1. Putative roles of reactive oxygen species (ROS) in the pathogenesis of pulmonary fibrosis. Fibrotic stimuli of unknown origin are thought to create an imbalance between oxidant production and antioxidant protection, resulting in the accumulation of ROS. Widespread effects on epithelium, myofibroblasts, and inflammatory cells may lead to destruction of the normal lung architecture via excessive apoptosis of alveolar epithelial cells and/or the development of resistance to apoptotic cell death in fibroblasts/myofibroblasts. The accumulation of these effects results in the development of end-stage fibrosis.

and the underlying fibroblasts undergo apoptosis. When the oxidative damage is sustained and/or the repair process hindered, the fibroblasts persist and ECM proteins continue to accumulate, resulting in disrupted lung architecture and impaired gas exchange (Fig. 1).

Several studies document increased levels of oxidative stress as well as reduced or otherwise compromised antioxidant protection in individuals with IPF. One study indicated that pulmonary inflammatory cells isolated from bronchoalveolar lavage (BAL) fluid of IPF patients generated higher levels of oxidants than those from control patients (2). Conversely, levels of the critical ROS scavenger glutathione, as well as antioxidant enzymes such as superoxide dismutases and catalase, are reduced in the alveolar lining fluid of affected patients (18). As a result, the increased levels of oxidants present during the development and progression of IPF may alter the integrity of the ECM or initiate/perpetuate damage to lung cells.

APOPTOTIC SIGNALING PATHWAYS

Apoptosis, the best-characterized process of programmed cell death, manifests through a highly conserved signaling pathway (Fig. 2) that results in the efficient dismantling of the cell without triggering an innate immune response in the organism (reviewed in Ref. 6). Apoptosis functions in the adult organism to maintain cellular homeostasis. When homeostasis is compromised, by either too much or not enough apoptosis under a given condition, pathology results.

Central to the apoptotic pathway of cell death is the activity of a family of cysteine proteases termed caspases that specifically cleave a wide variety of substrate proteins at aspartic acid residues. Caspases normally exist as inactive zymogens that, when processed, are cleaved at two aspartic acid residues to yield a dimer. The subsequent association of two dimers forms the final tetrameric structure and yields an active enzyme. Two

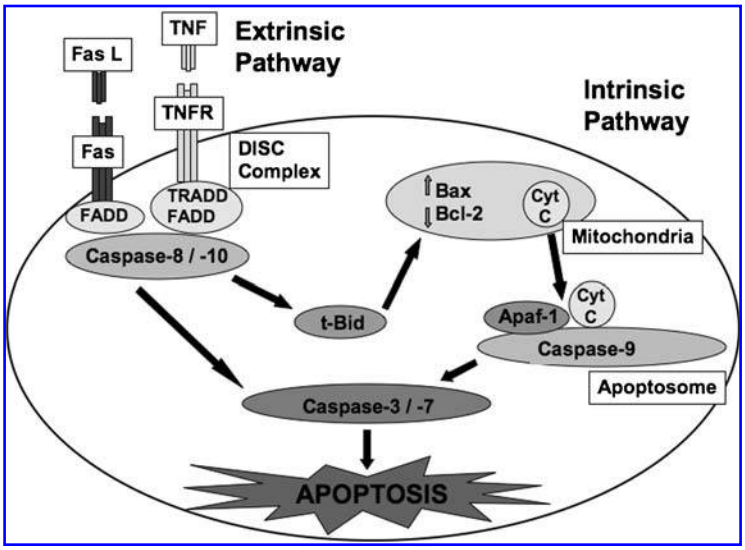

FIG. 2. The apoptotic signaling cascade. Both the intrinsic and extrinsic pathways of apoptotic signaling are thought to be involved in the death of lung cells during the development of IPF. The intrinsic apoptotic pathway is triggered when a stimulus that leads to an increase in the levels of pro-apoptotic Bcl-2 family members (*e.g.*, Bax) and/or a decrease in anti-apoptotic Bcl-2 proteins (*e.g.*, Bcl-2). This, often combined with a change in mitochondrial membrane potential, leads to the release of cytochrome c from the mitochondria. Cytochrome c, apaf-1, and caspase-9 assemble an apoptosome leading to the activation of caspase-9 and a cascade of activation events for the effector caspases, such as caspase-3 and caspase-7. The extrinsic pathway also leads to effector caspase activation via the binding of an extracellular ligand (*e.g.*, Fas, TNF-α) to transmembrane death receptors. Triggering of the death receptors leads to the formation of a DISC signaling complex associated with the cytoplasmic tail of the receptors. Assembly of the DISC results in the activation of caspase-8 (and often caspase-10) which, in turn, activates the effector caspases. Effector caspase activation ultimately leads to the dismantling and destruction of the cell.

overarching pathways of caspase activation have been described based on whether the initial apoptotic signal originates with the activation of cell surface receptors (*the extrinsic pathway*) or via intracellular sensors (*the intrinsic pathway*). A comprehensive review of these two pathways has been recently published (6), however a brief review of the essential signaling molecules is provided below.

The extrinsic pathway is triggered when members of the tumor necrosis factor-receptor (TNF-R) superfamily (including Fas/CD95 and DR 4/5) bind their ligands (FasL, TRAIL) in the extracellular space. Ligand binding stimulates the activation of these receptors that, in turn, recruit adaptor proteins to their cytoplasmic tails, starting with Fas-associated death domain (FADD). FADD acts as a platform onto which can assemble a death-inducing signaling complex (DISC) that includes a pro-caspase-8 and/or -10. Autocatalytic activation of pro-caspase-8 to its active form starts a cascade of proteolytic activations of downstream caspases, particularly caspase-3 and -7, which are responsible for the ultimate breakdown of the cell.

The intrinsic pathway is initiated by intracellular signals that activate proapoptotic Bcl-2 family members. Activation of proteins such as Bax and Bak overcomes the antiapoptotic activities of other Bcl-2 family members, including Bcl-2 itself. Upon the receipt of a death signal, Bax, for example, can oligomerize, insert into the mitochondrial membrane, and coordinate the release of cytochrome c from mitochondria. This activity, in turn, triggers apoptosome formation by directly activating apoptosis protease activating factor 1 (Apaf-1). The assembly of the apoptosome complex ensures that pro-caspase-9 is present in high concentration and proper orientation for its auto-activation. Activation of caspase-9 results in the subsequent activation of caspase-3 and -7.

OXIDATIVE STRESS-INDUCED APOPTOSIS

A growing body of evidence suggests that reactive oxygen species (ROS) can cause modifications or degradation of diverse cellular components including protein, lipid and DNA molecules (reviewed in Ref. 20). In addition, ROS can also function as important intracellular and intercellular signaling molecules that can affect gene expression, cell growth, and various forms of cell death (8). Morphological and biochemical characteristics of apoptosis, including caspase-3 activation, release of cytochrome c from the mitochondria, and DNA fragmentation have been observed in cell culture systems after treatment with compounds that produce ROS (5). Furthermore, upregulation of antioxidant defenses or antiapoptotic proteins, as well as down regulation of proapoptotic mediators, protect against ROS-mediated cell death.

A number of cell signaling pathways are thought to be involved in the transduction of the ROS-mediated 'death signal.' In particular, activation of the MAPK pathways often occurs upon exposure to ROS, with sustained activation of ERK1/2, JNK, and p38 MAPK (reviewed in Ref. 27). JNK is particularly interesting in this context since a recent study linked activation of this kinase with activation of the extrinsic apoptotic pathway, including death receptor activation and adaptor molecule recruitment (see below). Stimulation of receptor tyrosine kinases such as EGFR, PDGFR, and VEGFR (4) or activation of the PKC-mediated pathways (14) has also been associated with oxidant-induced apoptosis. Thus, it seems that generation of excessive ROS, when left unchecked, can cause extensive cellular damage that leads to cell death.

ALVEOLAR EPITHELIAL CELL DEATH—TOO MUCH APOPTOSIS

One of the earliest studies to detail apoptosis in IPF showed that bronchiolar and alveolar epithelial cells isolated from lung biopsy specimens from patients displayed significant numbers of DNA strand breaks (a hallmark of apoptosis) (15). Later studies showed that these apoptotic epithelial cells co-localized in areas of myofibroblasts accumulation and collagen deposition (23). On a intracellular level, studies have shown increased expression of proapoptotic proteins and decreased expression of antiapoptotic proteins in AECs isolated from IPF patients (17). Furthermore, factors produced by myofibroblasts isolated from IPF patients produced a number of factors, including angiotensin peptides that could induced apoptosis in alveolar epithelial cells *in vitro* (26).

Reactive oxygen species may be a primary mediators of alveolar epithelial cell death in IPF (Fig. 3) (2). Inflammatory cells, primarily macrophages and neutrophils, and also parenchymal cells such as myofibroblasts are all sources of ROS. These cells produce sufficient quantities of ROS to induce apoptosis in the nearby epithelial cells. In addition, many of the intracellular signaling pathways implicated in ROS-mediated apoptosis have also been shown to be active in the lungs of patients with IPF. For example, the activation of ERK is significantly decreased in epithelial cells but not fibroblasts of IPF lungs. In addition, activated JNK levels were increased in these same cells. Both of these increases were associated with the presence of apoptotic cells and the progression of fibrosis (29).

The extrinsic apoptotic pathway seems to be an important route to apoptotic cell death in IPF (reviewed in Ref. 7). In the late 1990s (11), Hara's group in Japan first described the role of the Fas—Fas ligand (FasL and its soluble form sFasL) pathway of apoptosis in the pathogenesis of IPF. Contemporary studies demonstrated that upregulation of Fas and FasL both in lung epithelial and BAL cells in mice were associated with excessive apoptosis and progressive pulmonary fibrosis. Later analyses of BAL fluid from IPF patients confirmed this result by showing increased levels of Fas in epithelial cells, macrophages, and neutrophils, whereas both sFas and Fas receptor was increased in lymphocytes. Furthermore, in thoracoscopic biopsy specimens from patients diagnosed with IPF, components of the Fas signaling pathway, including FADD and caspase-3, were upregulated and correlated with increased levels of apoptotic cell death.

EPITHELIAL TO MESENCHYMAL TRANSITION (EMT)

The interaction between epithelial and mesenchymal cells in the lung is essential for normal lung function. Studies in ani-

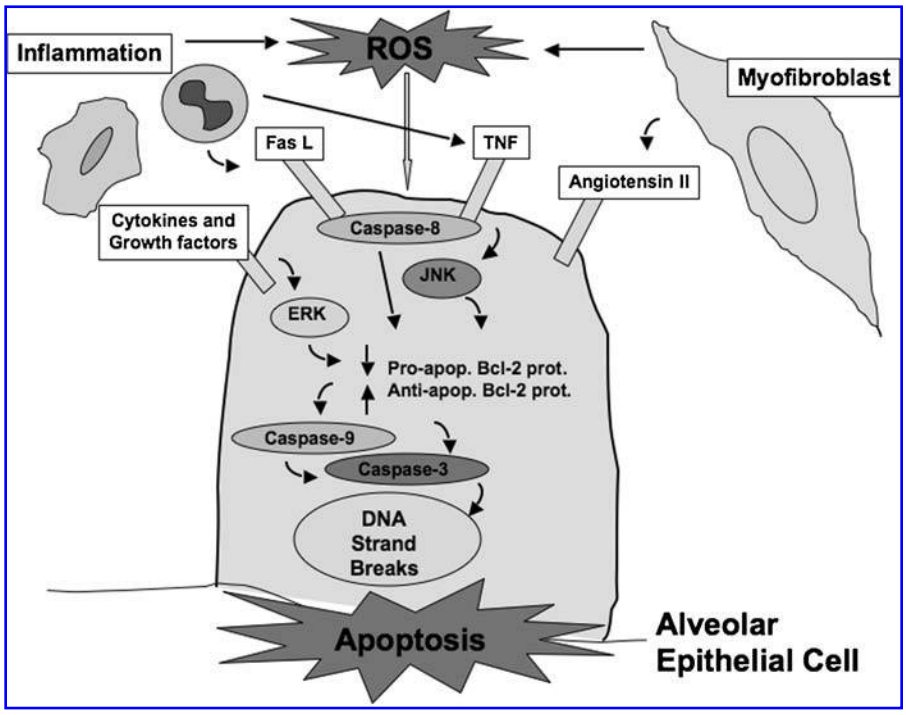

FIG. 3. Putative proapoptotic pathways and mediators involved in the destruction of alveolar epithelial cells in pulmonary fibrosis. Endogenous and exogenous mediators of apoptosis are thought to be involved in triggering the death of alveolar epithelial cells via both the intrinsic and extrinsic apoptotic pathways.

mal models suggest that miscommunication between these two cell types is a mechanism for the development of fibrotic lung disease (28). Induction of alveolar epithelial cell injury by adjacent myofibroblasts can be mediated, in part, by generation of ROS via TGF-β stimulation of the myofibroblast population. Moreover, the presence of TGF-β can enhance Fas-mediated AEC apoptosis. Also, TGF-β can stimulate alveolar epithelial cells to express a number of mesenchymal cell markers. This transition from epithelial to myofibroblastic phenotype may account for a significant amount of mesenchymal expansion in models of IPF. However, the lung microenvironment is also important, as isolated AECs will undergo apoptosis instead of EMT when cultured on matrix composed primarily of lamin and collagen. Thus, signaling mediators produced by myofibroblasts and the ECM can influence, either directly or indirectly, alveolar epithelial cell survival.

FIBROBLAST SURVIVAL—TOO LITTLE APOPTOSIS

Effective wound healing requires the removal of excess mesenchymal cells from the site of injury. In disorders characterized by pulmonary fibrosis, removal of these cells is delayed or does not occur. A recent theory regarding the pathogenesis of pulmonary fibrosis states that an acquired resistance to apoptosis in the myofibroblast population may account for the increased number of these cells present in fibroblastic foci (reviewed in Ref. 21). In fact, mesenchymal cells located in areas of fibroproliferation have been characterized as having an antiapoptotic phenotype (Fig. 4); (*i.e.*, the continuous activation of signaling pathways, such as the PI3K/AKT pathway, that promote cell survival). Several mechanisms have been proposed to account for the resistance of mesenchymal cells to apoptosis including specific genetic alterations in the mesenchymal cells themselves and dysregulated responses of these cells to the lung microenvironment. Thus, it may be that the acquisition of these prosurvival characteristics by mesenchymal cells is contributing to the overproliferation of fibroblasts and myofibroblasts in IPF.

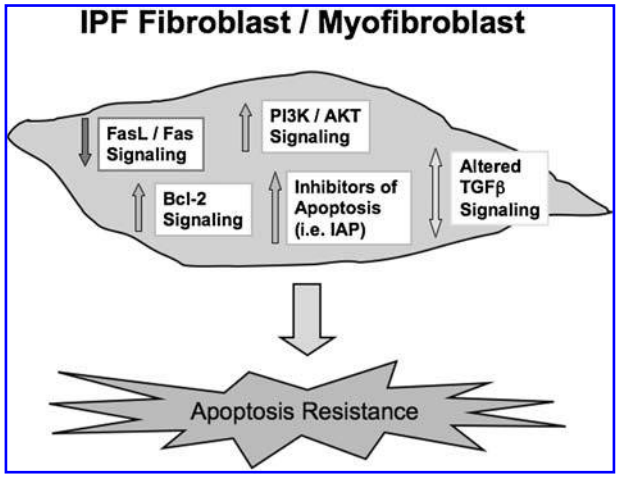

FIG. 4. Putative anti-apoptotic pathways and mediators involved in the survival of fibroblasts and myofibroblasts in pulmonary fibrosis. A number of anti-apoptotic signals are thought to be active in the fibroblasts/myofibroblasts of the fibrotic lung. Persistence of these apoptosis-resistant cells in the lungs during or after a repair event may lead to excessive deposition of extracellular matrix components, a hallmark of lung fibrosis.

Many studies using fibroblasts isolated from biopsy specimens from patients with IPF have demonstrated that these cells display dysregulated apoptotic signaling. Fibroblasts isolated from the lungs of IPF patients are more resistant to Fas-L-induced apoptosis as compared to fibroblasts isolated from normal lung (16). In addition, the resistance of these cells to apoptotic stimuli is accompanied by the upregulation of anti-apoptotic proteins such as IAP and FLICE-like inhibitor. However, these results may not constitute the whole story since other groups have shown that fibroblasts isolated from IPF patients exhibit reduced growth rates and increased rates of spontaneous apoptosis (19).

INFLAMMATORY CELL APOPTOSIS—THE ROLE OF THE MACROPHAGE

Fibrotic lung diseases such as IPF are often characterized by the presence of inflammatory cells including macrophages, neutrophils, and several classes of lymphocytes. Elevated levels of inflammatory cytokines have been noted both in animal models of lung fibrosis as well as BAL fluid from patients. Alveolar macrophages, in particular, may be important in the pathogenesis of pulmonary fibrosis, as the release of cytokines by these cells may serve to recruit additional inflammatory cells to the injured lungs (1) and that dysregulation of macrophage recruitment and/or elimination may play a key role in the progression and resolution of lung injury. It may be that prolonged survival of these cells supports the development of pulmonary fibrosis. This notion is supported by a recent study by Flaherty *et al*. (10), which described prolonged alveolar macrophage survival in the presence of increased ROS concentrations. Survival was augmented via a mechanism that involves sustained signaling of the PI3K/AKT pathway, similar to that noted in prolonged fibroblast survival.

The direct effect of alveolar macrophage apoptosis on lung injury was recently investigated by Wang *et al*. (24). This group showed that instillation of apoptotic macrophages into the rat lungs resulted in an increase in the number of apoptotic lung cells at later timepoints (24). In addition, the instillation of apoptotic macrophages induced an additional infiltration of 'healthy' macrophages, which in turn, also underwent apoptosis. It is interesting to note that macrophages ingesting apoptotic bodies release a number of soluble factors that trigger apoptosis of other lung cells (including alveolar epithelial cells) and/or promote fibrosis. This *in vivo* induction of apoptosis, termed secondary apoptosis, appears to set up a potent feed-forward loop, resulting in a disproportional amount of cell death in response to a given stimuli. Secondary apoptosis may have particular relevance to IPF, since the presence of the apoptotic cells themselves were sufficient to induce lung injury in the absence of

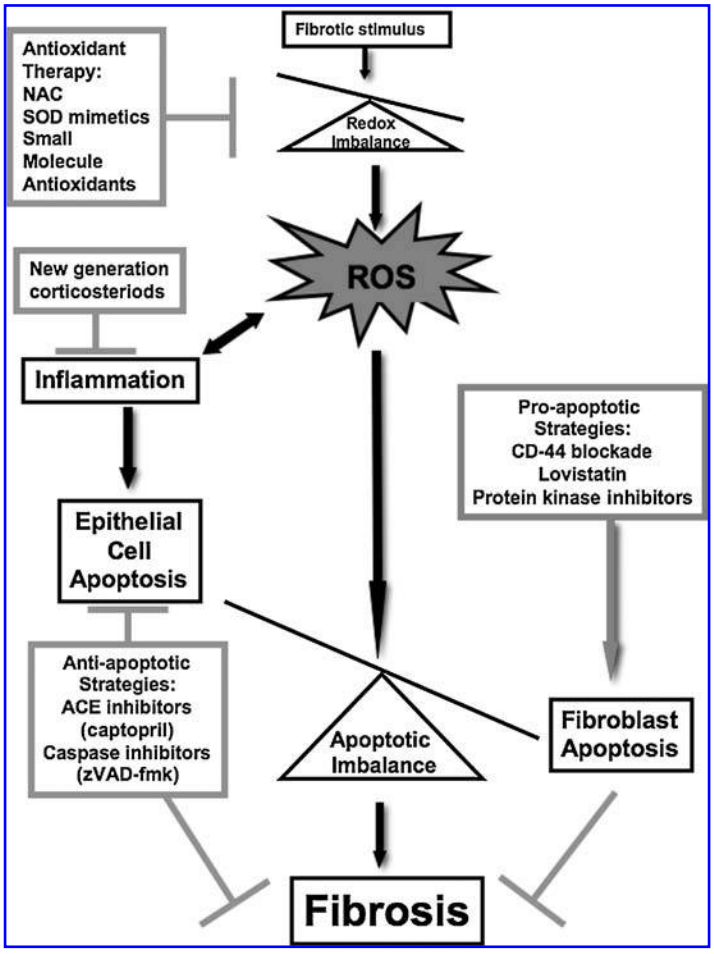

FIG. 5. Steps at which therapeutics may control apoptosis during the development of pulmonary fibrosis. Re-establishing the redox balance, inhibiting inflammation, inhibiting apoptosis in alveolar epithelial cells or macrophages, and promoting apoptosis in fibroblasts/myofibroblasts are strategies that are currently under investigation to be used as therapy in pulmonary fibrosis.

any other defined stimuli. This observation supports the idea that IPF may not result from ongoing presence of a fibrotic stimulus, but rather from a failure of the normal phagocytotic clearance mechanisms.

PHARMACOLOGIC INTERVENTION

Because the execution of apoptosis requires the activation of specific signaling pathways, this process holds great possibilities for the development of pharmacologic agents that target the enzymes and other proteins involved (Fig. 5). Several studies have demonstrated that administration of compounds with antioxidant properties can ameliorate pulmonary fibrosis in a number of model systems (13). Other routes of inhibition of apoptosis that have been examined in animal studies include new generation corticosteroids (3) and inhibitors of the rennin–angiotensin system. For example, captopril, an angiotensin-converting enzyme inhibitor, has been shown to inhibit the formation of lung fibrosis in bleomycin-treated mice (25). Studies examining the mechanism of captopril-mediated protection have shown that this compound inhibits Fas-mediated apoptosis of human alveolar epithelial cells, suggesting that abrogation of AEC death may be a viable mechanism for the prevention of pulmonary fibrosis. In fact, the direct inhibition of the caspase enzymes by administration of the pan-caspase inhibitor zVAD-fmk in rats showed that these animals had a significant reduction in collagen deposition and epithelial apoptosis in response to bleomycin.

CONCLUSIONS

Restoration of an intact epithelium is important in the resolution of pulmonary fibrosis. Accumulating evidence suggests that the fibrogenic response is driven by the death of alveolar epithelial cells and the subsequent loss of AEC-produced mediators of fibroblast proliferation. The loss of the physical barrier between interstitial cells and the alveolar space may promote fibroblast migration while the acquisition of an apoptosis-resistant phenotype in fibroblasts and myofibroblasts may promote the expansion of this cell population. It is clear that gaining a greater understanding of the biochemical mechanisms, epigenetic changes, and molecular and cellular influences on the stimulation or inhibition of apoptosis in the fibrotic lung is necessary for the development of novel targeted therapies for idiopathic pulmonary fibrosis.

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ABBREVIATIONS

AEC, alveolar epithelial cell; APAF-1, apoptosis protease activating factor-1; bcl-2, b-cell lymphoma protein-2; bax, bcl-2 associated X protein; bak, bcl-2 homologous antagonist-killer protein; BAL, bronchoalveolar lavage; DISC, death-inducing signaling complex; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; ECM, extracellular matrix; FADD, fas-associated death domain; IPF, idiopathic pulmonary fibrosis; IAP, inhibitor of apoptosis; IL, interleukin; MAPK, mitogen-activated protein kinase; PI3K, 1-phosphatidylinositol-3 kinase; PDGFR, platelet-derived growth factor receptor; PKC, protein kinase C; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand; VEGFR, vascular endothelial growth factor receptor.

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